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Recent advances in immobilized enzyme-based analytical methods, e.g., the cholinesterase-based water monitor "CAM" (cholinesterase antagonist monitor), have proved useful in the detection of organophosphate and carbamate pesticides. This work has now been extended to the detection of five high molecular weight chlorinated hydrocarbons at the parts-per-million (ppm) level in a water/methanol matrix. In the new method being developed, the lactate dehydrogenase (LDH) catalyzed reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{[inhibitor]}]{\text{LDH}} \text{lactate} + \text{NAD}^+$$

is used to detect the presence of chlorinated hydrocarbons, which reduce the rate of the reaction by inhibiting the enzyme. The inhibition is reversible and is competitive with respect to the substrate pyruvate and the coenzyme nicotinamide adenine dinucleotide (NADH). The rate of reaction is readily measured potentiometrically by following the pH change. To date, the method has proved effective in detecting aldrin, toxaphene, Aroclor 1242, DDT, and pentachlorophenol. Nonchlorinated analogs, e.g. phenols, are detected only at orders of magnitude higher concentrations. The method has not yet been evaluated on environmental samples.

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ENZYME-BASED DETECTION OF CHLORINATED HYDROCARBONS[1]

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ABSTRACT

Recent advances in immobilized enzyme-based analytical methods, e.g., the cholinesterase-based water monitor "CAM" (cholinesterase antagonist monitor), have proved useful in the detection of organophosphate and carbamate pesticides. This work has now been extended to the detection of five high molecular weight chlorinated hydrocarbons at the parts-per-million (ppm) level in a water/methanol matrix. In the new method being developed, the lactate dehydrogenase (LDH) catalyzed reaction:

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INTRODUCTION

Simple and robust field methods for the detection of chlorinated hydrocarbons are needed for determining the boundaries of an area targeted for decontamination, waste site management, and the rapid detection of spills. Recent advances in the development of analytical methods based on immobilized enzymes have proved useful in the detection of pesticides. Specifically, CAM devices have been developed by Midwest Research Institute under EPA Contracts 68-01-0038 and 68-03-0299 for monitoring organophosphate and carbamate pesticides in water. In the present study, "CAM-type" methods have been developed for the detection of high molecular weight chlorinated hydrocarbons. Based on an extensive literature review, lactate dehydrogenase was chosen to replace cholinesterase. The reaction:

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{[inhibitor]}_2]{\text{LDH}} \text{lactate} + NAD^+$$

was found to be useful in the detection of chlorinated hydrocarbons, which reduce the rate of the reaction. The inhibition of LDH by these high molecular weight chlorinated hydrocarbons is reversible and is competitive with respect to pyruvate and the coenzyme NADH. The experimental work described below shows that five chlorinated hydrocarbons can be detected at 10^{-4} moles/liter (M) and 10^{-5} M, and two are detectable at concentrations of 10^{-6} M by this reaction. Phenol has been tested and does not interfere with the reaction and it is expected that other nonchlorinated hydrocarbon analogs also will not interfere. Either pH or potentiometric detection of the coenzyme can be used. Since the pollutant inhibition of the LDH is reversible, the LDH enzyme can be re-activated and re-used if it is immobilized on a suitable support medium. Re-use of the enzyme enhances the cost-effectiveness of the method. Enzyme immobilization is essential in instrumentation designed for field use, reducing both complexity and cost per test.

LDH INHIBITION STUDIES

The results of the experimental inhibition studies are shown in Table 1, which summarizes some seventy inhibition assays on seven representative test compounds. The inhibition results are consistent with the published literature where overlap of data exists, e.g., for aldrin (Hendrickson and Bowden, 1976). LDH sensitivity to chlorinated hydrocarbons approximately parallels the relative toxicity of the compounds. While the 2,4,5-T inhibition, 12% at the highest concentration tested, is below the level of significance, inhibition is in fact expected on theoretical grounds.

TABLE 1. INHIBITION OF LACTATE DEHYDROGENASE

TEST COMPOUND	% INHIBITION* AT SEVERAL CONCENTRATIONS		
	10^{-4} M	10^{-5} M	10^{-6} M
CYCLODIENE INSECTICIDES			
Aldrin	100**	58	29
Toxaphene	100**	40	13
CHLORINATED ETHANE DERIVATIVES			
DDT	100**	22	6
POLYCHLORINATED BIPHENYLS			
Aroclor 1242	100**	22	0
POLYCHLORINATED PHENOLS			
Pentachlorophenol	74	23	0
CHLOROPHENOXY DERIVATIVES			
2,4,5 Trichlorophenoxy Acetic Acid	12	0	-
MISC. CHLORINATED HYDROCARBONS			
Lindane	0	0	-

* Percent inhibition was calculated by assigning 100% activity to a methanol (20%) control containing no test compound.

** Undissolved test compound present.

The "100**" values require an explanation. If inhibitor that is undetected and undissolved is present at the lower concentrations tested, then the data in Table 1 could overestimate inhibition effects projected for field devices. When undissolved inhibitor is present, coprecipitation of enzyme and inhibitor has been observed. This effect mimics inhibition by reducing the active enzyme concentration and has been extensively documented by Pocker, 1971. Undissolved inhibitor, if present in field samples, will react differently with the immobilized enzyme preparations of field devices than with the free enzyme used in the inhibition studies. For this reason, the distinction between coprecipitation and inhibition is of more than academic interest. The possibility of coprecipitation was ruled out by mechanistic studies of the type illustrated in Figure 1. Linear double-reciprocal plots of initial reaction velocity versus substrate concentration are only consistent with reversible inhibition and are inconsistent with the occurrence of coprecipitation.

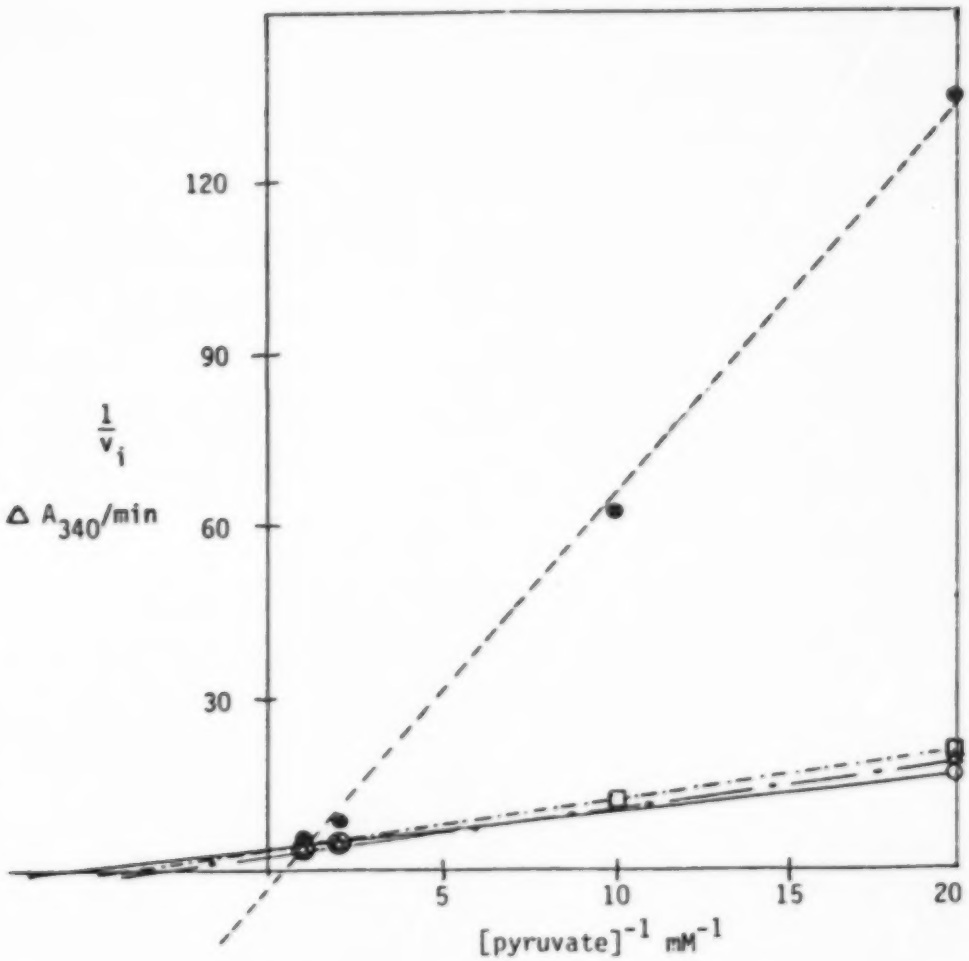

FIGURE 1. The inhibition of LDH by Arochlor 1242. Lineweaver-Burk double reciprocal plot of the inhibition with respect to pyruvate. Experimental conditions: [NADH] = 0.22 mM; [Arochlor 1242] = (o) 0 mM; (Δ) 0.005 mM; (□) 0.01 mM; (●) 0.05 mM. The reaction was run in 0.1 M phosphate buffer-methanol (80:20; v/v) at pH 7.5, at 25°C.

INTERFERENCES

Nonhalogenated aromatic pollutants, excepting phenols and amines, are even less soluble than their chlorinated analogs, so that interference is unlikely. Furthermore, interference is not expected on structural grounds. Phenol was tested and exhibited no significant inhibition at concentrations below 0.1 M.

The organophosphate pesticide, Diazino , and the carbamate, Sevin, inhibited LDH only at concentrations greater than 0.001 M. This indicates that cholinesterase (CAM) and LDH may have complementary detection capabilities toward Category X_1 organic hazardous substances. The LDH method appears to selectively detect high molecular weight chlorinated hydrocarbons, while the CAM method detects organophosphate and carbamate pesticides.

Cyanide tends to inhibit most enzymes. Concentrations as high a 0.01 M were required to exhibit 50% inhibition. The LDH method is hence unusually insensitive to toxic levels of cyanide. On the other hand, heavy metal ions, such as free mercuric ion (Hg^{+2}) showed complete inhibition consistent with the presence of sulfhydril (-SH) groups on the enzyme. Use of a suitable chelating agent will probably be necessary to suppress interference in environmental applications where high levels of heavy metal ions are present.

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1. Category X hazardous substance refers to those 21 organic compounds designated in 40 CFR 117.3 as having a reportable quantity of 1 pound.

DETECTION METHODS

In the laboratory work, the rate of the reaction was followed by spectrophotometrically determining the absorbance of the reaction mixture at the 340 nm peak of NADH. However, this detection method is not particularly suitable for field use. Natural waters tend to have background absorption, and to foul optical surfaces. Furthermore, optical methods of detection are relatively expensive. Two cheaper alternatives are available, potentiometric detection (monitoring of the $[NADH]/[NAD^+]$ couple), and pH detection (the reaction consumes hydrogen ion). Both methods are relatively insensitive to interference, although high concentrations of oxidants (e.g., chlorine) can distort potentiometric detection, just as high concentrations of acids and bases can distort pH detection. Due to time constraints, only one of the two methods (pH detection) could be examined in this study.

Since the reaction consumes hydrogen ion, the pH of the reaction mixture should increase with time. The extent of the pH increase depends upon the buffering capacity of the reaction mixture. When the reaction mixture was titrated with hydroxide, addition of 10^{-5} M OH^- at a pH initially equal to 7 produced a 1-unit pH change, rather than the 2-unit change expected in the absence of buffer. The difference is traceable to the buffering effect of impurities in the commercial enzyme preparation. As shown in Figure 2, when the reaction mixture (primarily sodium acetate) is titrated in the absence of enzyme, a far sharper pH change is observed. (A sodium acetate buffer limits pH decrease, but has no effect on pH increase.) Nevertheless, as shown in Figure 3, the

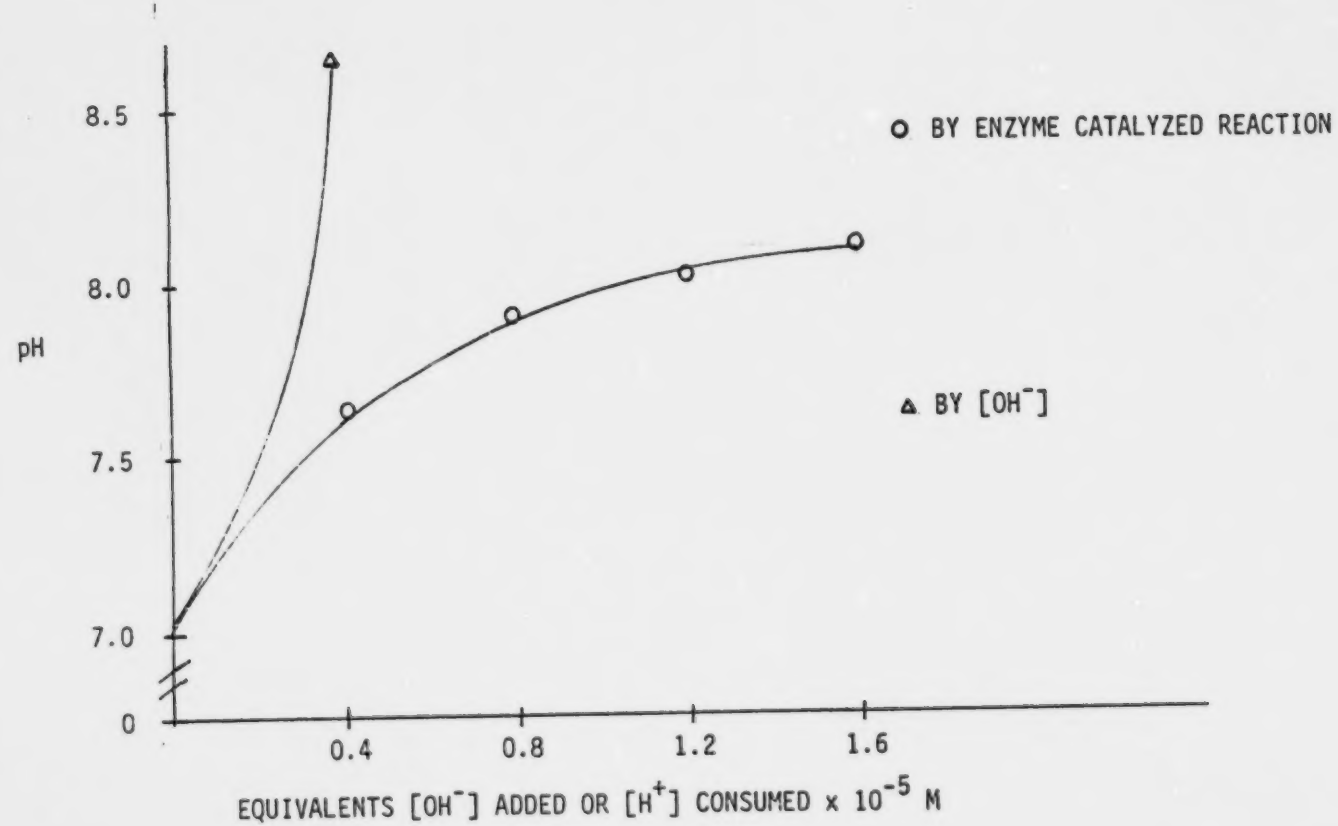

FIGURE 2. Titration of reaction mixture

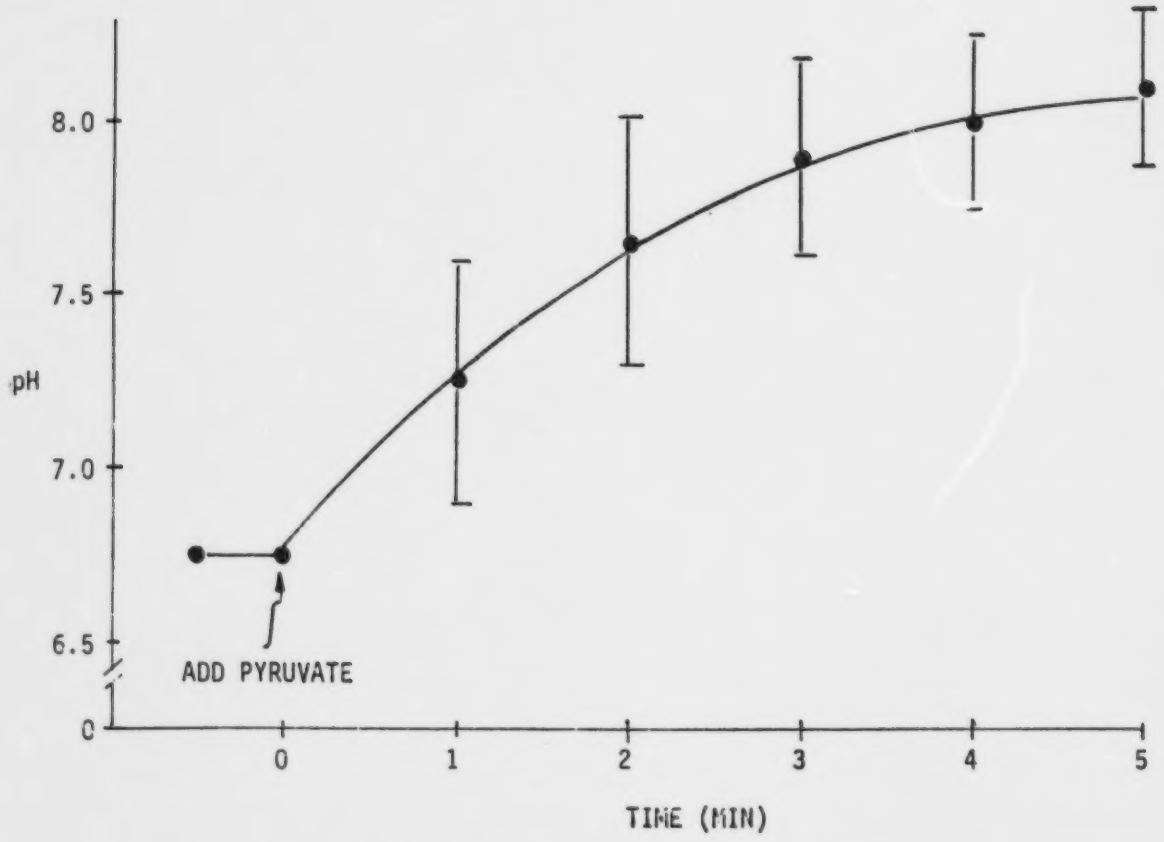

FIGURE 3. pH detection of the LDH catalyzed reaction

buffering effect of the enzyme impurities is not so great as to eliminate pH detection as a viable method. In the presence of high concentrations of a chlorinated hydrocarbon, the pH change would be zero; the pH change actually observed, in excess of 1 unit, clearly indicated the absence of inhibitors.

We conclude from these results that a 0.001 M acetate buffer in 20% methanol solution supports a relatively robust detection scheme. At this pH and methanol concentration, the enzyme activity is about 25% greater than the standard LDH assay, and the solution is well buffered against normal acidic waters. The only potential problems would occur in waters that are more basic than pH 7, such as sea water or brackish water samples. Potentiometric detection of $[NADH]/[NAD^+]$ could probably be used in such circumstances.

LDH IMMOBILIZATION

Immobilization of the LDH enzyme is a necessary step toward developing robust, cost-effective field methods. Many different types of immobilization techniques have been successfully applied to LDH. Several look quite promising for developing continuous flow applications and field detector designs. However, each immobilization method must be examined with care, because immobilizing an enzyme can also reduce its activity and/or reduce its sensitivity to inhibition. Among the various LDH immobilization techniques, covalent bonding methods (Guire, 1974) employing a spacer molecule between the enzyme and its support are best

suited to reproducing the free enzyme environment in an immobilized preparation. As of this writing, this technique has not been attempted, although it is clearly recommended for future method development.

CONCLUSIONS

An LDH enzyme-based detector has been developed that can detect at least five chlorinated hydrocarbons at concentrations of 10^{-4} to 10^{-6}M in a 20% methanol/water solution. Based on literature reviews, it is thought that the detector will also be sensitive to other high molecular weight chlorinated hydrocarbons. Phenol test data and analysis of the literature indicate that interference by nonchlorinated analogs will not occur. The simplicity and rapidity of the method indicates promise for field applications. The inhibition of the LDH by chlorinated hydrocarbons can be monitored either by pH or potentiometric changes in the reaction mixture and this provides flexibility that also enhances the potential of the detection method for field use.

Although the LDH enzyme-based detector has potential for field application, more work is required in several areas, including (1) development of an extraction/concentration procedure (with an enzyme-compatible solvent) to preceed the use of the LDH detection method

for chlorinated hydrocarbons that have very low water solubility, (2) immobilization of the LDH enzyme on a suitable support to make re-use of the enzyme possible and to simplify the detection procedure, and (3) evaluation of the detection method's sensitivity to potential interferents in environmental samples.

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